Evaluation of Cage Designs and Feeding Regimes for Honey Bee (Hymenoptera: Apidae) Laboratory Experiments

SHAO KANG HUANG,[1] TAMAS CSAKI,[2] VINCENT DOUBLET,[3] CLAUDIA DUSSAUBAT,[4] JAY D. EVANS,[5] ANNA M. GAJDA,[6] ALEX GREGORC,[7] MICHELE C. HAMILTON,[5] MARTIN KAMLER,[8] ANTOINE LECOCQ,[9] MUSTAFA N. MUZ,[10] PETER NEUMANN,[11,12] ASLI ÖZKIRIM,[13] AYGÜN SCHIESSER,[13] ALEX R. SOHR,[14] GINA TANNER,[11,12] CANSU ÖZGE TOZKAR,[15] GEOFFREY R. WILLIAMS,[11,12] LYMAN WU,[14] HUOQING ZHENG,[16] AND YAN PING CHEN[5,17]

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ABSTRACT The aim of this study was to improve cage systems for maintaining adult honey bee (*Apis mellifera* L.) workers under in vitro laboratory conditions. To achieve this goal, we experimentally evaluated the impact of different cages, developed by scientists of the international research network COLOSS (Prevention of honey bee COlony LOSSes), on the physiology and survival of honey bees. We identified three cages that promoted good survival of honey bees. The bees from cages that exhibited greater survival had relatively lower titers of deformed wing virus, suggesting that deformed wing virus is a significant marker reflecting stress level and health status of the host. We also determined that a leak- and drip-proof feeder was an integral part of a cage system and a feeder modified from a 20-ml plastic syringe displayed the best result in providing steady food supply to bees. Finally, we also demonstrated that the addition of protein to the bees' diet could significantly increase the level of vitellogenin gene expression and improve bees' survival. This international collaborative study represents a critical step toward improvement of cage designs and feeding regimes for honey bee laboratory experiments.

KEY WORDS *Apis mellifera*, cage, laboratory testing, COLOSS, honey bee

Approximately 70% of the world's most valuable crops used directly for human consumption depend on insects for pollination, contribute an estimated €153 billion to the global economy, and represent 9.5% of the entire world agricultural production used for human food in 2005 (Gallai et al. 2009). European honey bees, *Apis mellifera* L., remain the most economically valuable pollinators of agricultural crops worldwide (Klein et al. 2007). However, honey bee populations have been declining in European and North America during last decades at an alarming rate (Pettis and Delaplane 2010; Potts et al. 2010; vanEngelsdrop and Meixner 2010; Williams et al. 2010, 2013).The problem of bee declines has been exacerbated by colony collapse disorder (CCD), a mysterious malady that initially affected bee populations during the winter of 2006–2007 in the continental United States, and later other parts of the world as well (Neumann and Carreck 2010, Ratnieks and Carreck 2010, Williams et al. 2010, Dainat et al. 2012a). Researchers have been searching for factors and mechanisms that underlay colony losses, with the intent to develop appropriate strategies to reduce bee losses and improve bee management. Multiple factors such as pathogens, parasites,

[1] College of Bee Science, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, P.R. China.
[2] Szent István University, Institute for Wildlife Conservation, 2101 Gödöllö, Páter K. u. I., Hungary.
[3] Institute for Biology, Martin-Luther-University, Halle-Wittenberg, Hoher Weg 8, D-06099 Halle (Saale), Germany.
[4] INRA, UMR Abeilles et Environment, Laboratoire Biologie et Protection de [lapos]abeille, Site Agroparc, 84914 Avignon, France.
[5] USDA–ARS Bee Research Laboratory, BARC-East Bldg. 306, 10300 Baltimore Ave., Beltsville, MD 20705.
[6] Warsaw University of Life Science, Faculty of Veterinary Medicine, Ciszewskiego 8, 02-786 Warsaw, Poland.
[7] Agricultural Institute of Slovenia, Hacquetova 17, 1001 Ljubljana, Slovenia.
[8] Bee Research Institute at Dol, Dol 94, 252 66 Libcice nad Vltavou, Czech Republic.
[9] Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C., Denmark.
[10] Faculty of Veterinary Medicine, Department of Parasitology, University of Mustafa Kemal, Antakya-Hatay-Turkey 3100.
[11] Swiss Bee Research Centre, Agroscope Liebefeld-Posieux Research Station ALP-HARAS, Bern, Switzerland.
[12] Institute of Bee Health, Vetsuisse Faculty, University of Bern, Bern, Switzerland.
[13] Department of Biology, Bee Health Laboratory and Bee and Bee Products Research and Application Center, Hacettepe University, 06800 Beytepe-Ankara-Turkey.
[14] Department of Biology, University of Maryland, College Park, MD 20742.
[15] Department of Biology, Middle East Technical University, Ankara 06800, Turkey.
[16] College of Animal Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, P.R. China.
[17] Corresponding author, e-mail: judy.chen@ars.usda.gov.

malnutrition, pesticide residues, and loss of genetic diversity, working singly or synergistically, have been proposed as causal agents of colony losses (vanEngelsdorp et al. 2009, vanEngelsdrop and Meixner 2010). However, to date, studies have been unable to prove a causal relationship between a particular agent and the disorder effect, highlighting a need for further investigations to resolve unanswered questions.

To gain a better understanding of how various factors affect bees and how these factors interact with each other to affect the abundance and performance of honey bees, researchers often need to conduct a wide range of experiments to manipulate the level of independent variables, and then measure the responses. Knowledge of the possible impact that individual factors may exert on honey bee behavior, physiology, and overall colony health and survival can be aided tremendously by the ability to maintain bees under laboratory conditions. Laboratory experiments may lead to large-scale field experiments, and ultimately to innovative solutions to the bee health problems. One key element for appropriate maintenance of bees under a controlled environment to collect variety of biological data is the development of a containment system that is capable of providing an adequate environment for bees with ample food supply available that mimic colony conditions as closely as possible. During recent decades, numerous methods for caging and observing adult honey bees in the laboratory have emerged (Rinderer and Baxter 1978; Crailsheim and Stolberg 1989; Cremonz et al. 1998; Iwasa et al. 2004; Evans et al. 2009; DeGrandi-Hoffman et al. 2010; Williams et al. 2012, 2013). These containment systems, made from different materials including wood, plastic, glass, Plexiglas, and stainless steel, have been used successfully for facilitating studies on honey bees under defined laboratory conditions. For example, Rinderer and Baxter (1978) examined the effect of group size on longevity and hoarding behavior in a laboratory cage. Evans et al. (2009) developed a single-use cage that was made of a plastic cup and used for laboratory experiments by exposing adult honey bees to pathogens and other factors that impact their health. Within these cups, adult honey bees had a median survival of 36 d, and a maximum of 60+ days. DeGrandi-Hoffman et al. (2010) used Plexiglas cages to study the effects of protein nutrition on hypopharyngeal gland development and virus loads. The effects of adding proteins to caged bees were also reported previously (Crailsheim and Stolberg 1989, Cremonz et al. 1998). Filipovic-Moskovljevic estimated the minimum number of worker bees necessary for performing certain activities (Filipovic-Moskovljevic 1972). Although all of the cage designs displayed advantages of cost efficiency, easy handling, and avoidance of biased samples, differences in properties such as shape, size, and building material may impact experimental results. COLOSS (Prevention of honey bee COlony LOSSes) is an international research network that is composed of >300 partners from >60 countries that work together to better understand bee health at a global level and develop internationally recognized methods in honey bee research to promote comparison among results worldwide (Williams et al. 2012). It was determined during the COLOSS workshops in Bern, Switzerland, in 2009, Bologna, Italy, in 2010, and Istanbul, Turkey, in 2012 that an improved method for maintaining honey bees in cages is required, as the existing systems, although adequate to meet the needs of individual research groups, nonetheless vary considerably in practice and produce results that may not be comparable across different laboratories. For this purpose, an international working group for evaluating common cage systems for honey bee in vitro studies was formed during the COLOSS workshop in Istanbul. We screened various cage designs and feeders for effects on honey bee physiology and survival. Further efforts were made to develop an optimized cage system by combining the best design and diet in the hope of establishing a reference method for standardization of honey bees in hoarding cages.

Materials and Methods

Cages and Feeders. A variety of cages and feeders from multiple international research groups were mailed to the U. S. Department of Agriculture–Agricultural Research Service (USDA–ARS) Bee Research Laboratory (BRL) in Beltsville, MD, for evaluation. Cages used for evaluation and their corresponding number designations are shown in Fig. 1. Each cage was equipped with one or two feeders, which were filled with a 1:1 sucrose–water solution and inserted into the cage to provision bees ad libitum. Each feeder was inserted on top of its respective cage with an opening (≈1 mm in diameter) toward the center of the cage so that honey bees could feed. The feeders were made from plastic syringe tubes, plastic transfer pipettes, and plastic or glass bottles that were easy to remove from the cages; they were transparent to allow the solution inside to be viewed. Six different feeders associated with specific cages designs are shown in Fig. 2A.

Feeder-1, which was used in Cage-3 and Cage-11, was made of a plastic transfer pipette, but the entire arm of a straight narrow plastic transfer pipette was cut off and the opening near the bulb was ≈4 mm in diameter (Fig. 2). Feeder-2, which was used in Cage-4, was modified from a 20-ml plastic syringe by cutting off the Luer lock tip to prevent formation of air bubbles at the tip that might interfere with bee feeding. Feeder-3, equipped in Cage-5 and Cage-6, was made of a 20-ml plastic vial. Owing to the difficulty in obtaining an identical glass vial for Cage-8 during the course of the experiment, Feeder-4 was replaced with a 20-ml plastic vial, but followed the original lid design as Cage-8 (Fig. 1). Feeder-5, used in Cage-2, was made from a 5-ml Falcon (Thermo Fisher, Rockville, MD) round-bottom tube, and Feeder-6, used in Cage-7, Cage-9, and Cage-10, was made from a 15-ml Falcon conical tube. Feeders made from vials had a removable lid for filling and cleaning, and drainage holes (≈1 mm in diameter) were drilled into each lid,

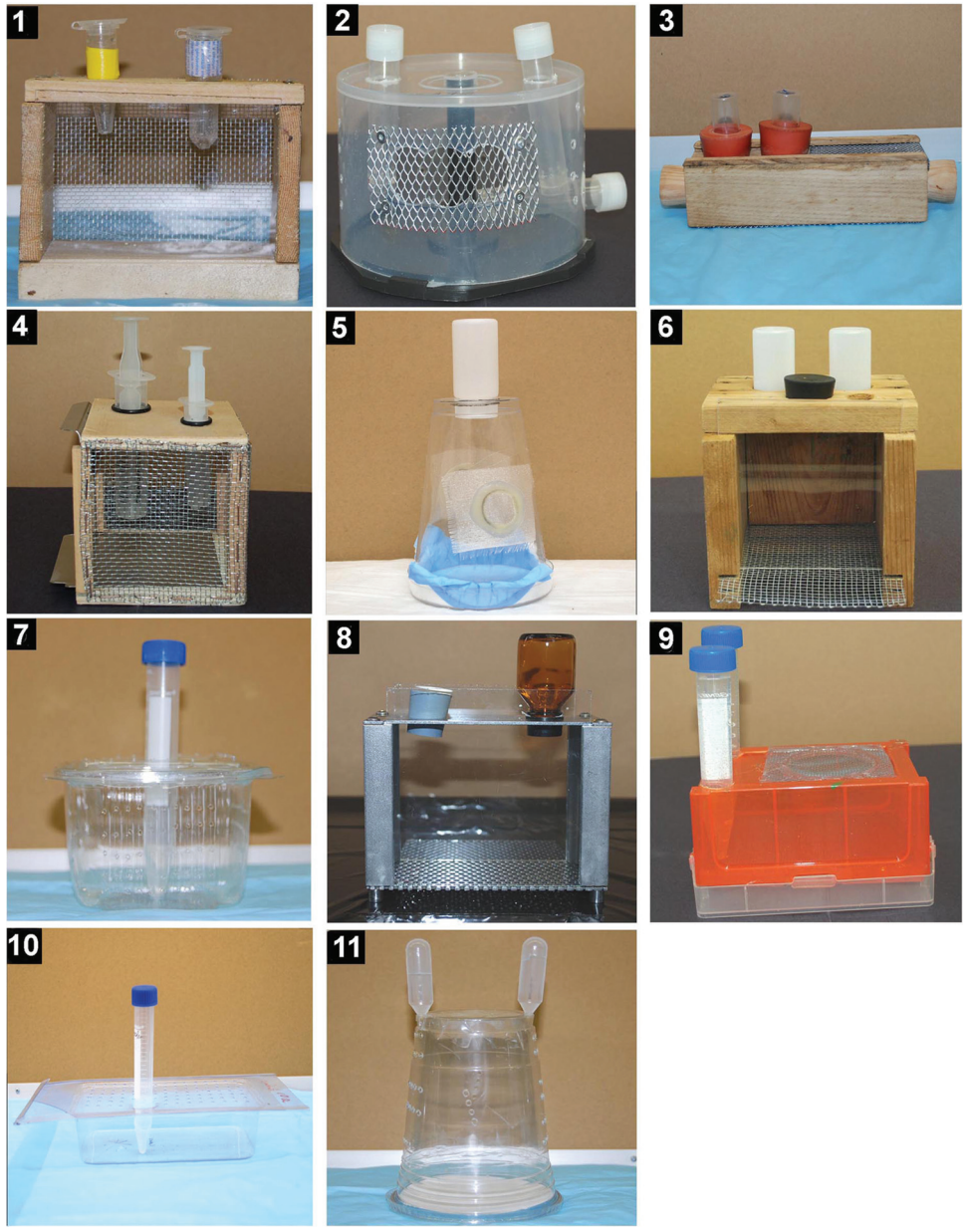

Fig. 1. Cages evaluated for honey bee in vitro experiments. Cage numbers are given in the upper left corner of each image.

while holes were drilled around the bottom of the tubes in Feeder-5 and Feeder-6. Owing to the small volume of Feeder-1 (1.5–2 ml), it was not included for further analysis.

Honey Bees. Honey bees (predominantly *A. mellifera ligustica*) were obtained from two different USDA Bee Research Laboratory apiaries in Beltsville, MD. Combs with capped worker brood close to emergence were removed from four field colonies, placed in mesh-walled cages, and incubated in an insect growth chamber at 34 ± 1°C and 55 ± 5% relative humidity (RH) overnight. Emerging adult worker bees were collected and pooled on the following day.

Cage and Feeder Evaluation. The experiment was carried out from May to September of 2012. With the exception of Cage-3, which had only 10 bees introduced because of its smaller size, 30 newly emerged bees were randomly selected and transferred to each bee-rearing cage. Each cage was replicated three times. All cages were maintained in an insect growth chamber (31 ± 1°C, 55 ± 5% RH, and dark). Feeders were refilled with fresh sucrose syrup every 3 d. Bee mortality was checked every

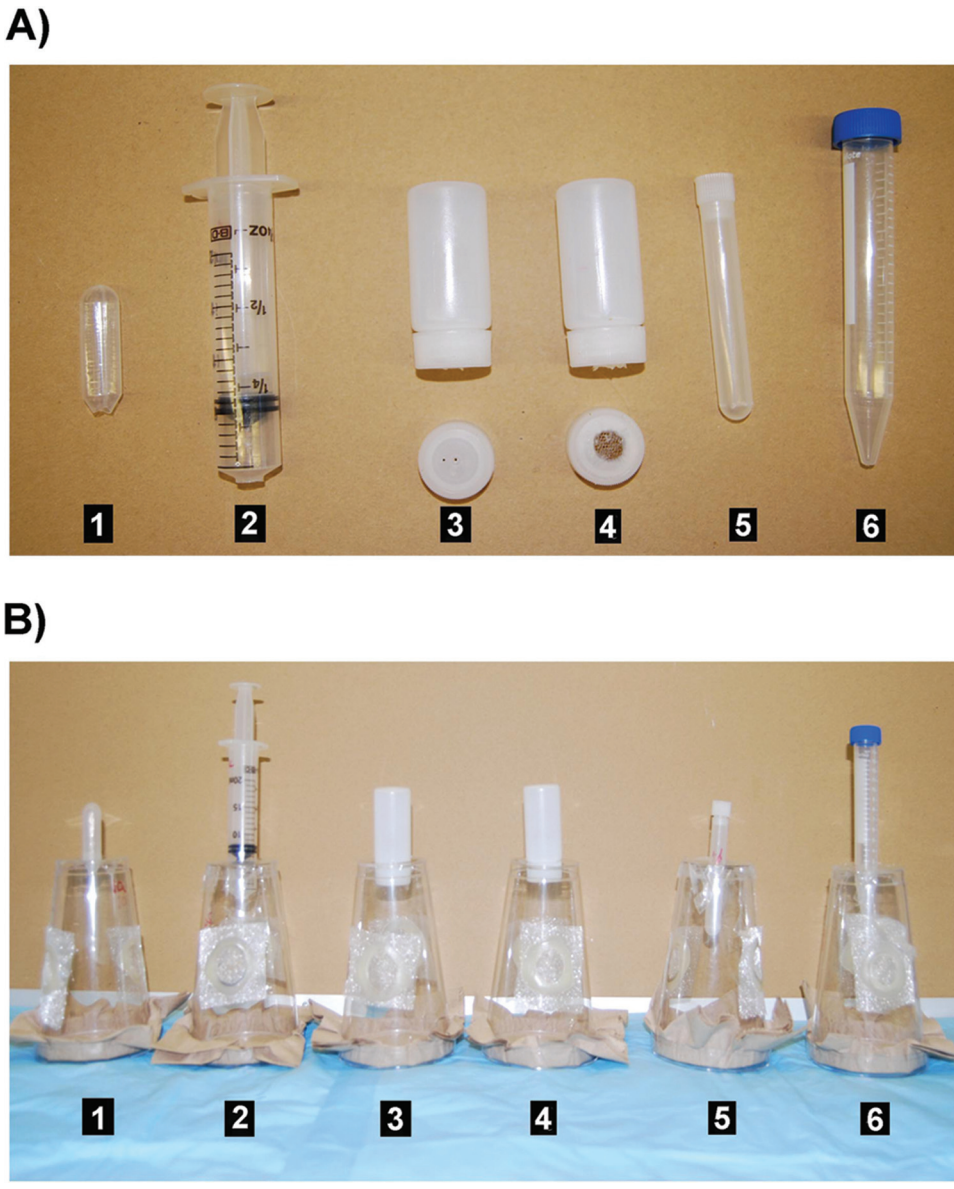

Fig. 2. (A) Six feeders used with the 11 cages. Feeder number is given below each feeder. (B) Different feeders that were individually inserted into Cage-5 for evaluation.

day until 100% mortality was reached for every cage. Dead bees were removed daily.

To compare the effects of feeder designs on the survival of caged bees, feeders were evaluated by using the same type of the cage but with different feeders. Cage-5 (Evans et al. 2009), which had displayed the best performance in terms of providing the longest survival for bees during our cage evaluation, was chosen as a reference cage for evaluation of various feeder designs. Six different feeders (Fig. 2A) were inserted over the top of Cage-5 individually (Fig. 2B). Following the same design for cage evaluation, 30 freshly emerged bees were introduced into each cage and bee mortality was checked every day until there was a group that reached 100% mortality. Each feeder was replicated three times and the evaluation was repeated four times.

In addition, a paper towel was placed under the bottom of the cage for absorbing possible drips from the feeders (Fig. 2B) and was weighed before a feeder was placed into the cage. After 3 d, the old paper towel was replaced with a new towel, and weighed. The quantity of sucrose syrup that leaked was calculated in grams based on the difference between the weights of each paper towel with and without absorbed syrup. The measurements were taken at 3-d intervals.

Deformed Wing Virus and Vitellogenin Quantification. To compare the effects of different cage designs on the general health of honey bees, the titer of

deformed wing virus (DWV) and expression level of vitellogenin (Vg) in bees from different cages were compared. DWV is one of the most common honey bee viruses and generally persists as a subclinical infection (Chen and Siede 2007).It can be activated to cause symptoms and illness in immune-compromised hosts and therefore serves as a significant marker for colony mortality (Dainat et al. 2012b). Vg, a precursor protein of egg yolk, has been a popular biomarker of general robustness of honey bees (Amdam et al. 2005, Simone et al. 2009). To do this, the newly emerged worker bees were transferred into individual cages following the same design for cage evaluation; when bee mortality in the cages reached 50%, live bees were collected from individual cages and immediately frozen in a −80°C freezer for subsequent molecular analysis.

Total RNA was isolated from individual bees collected from cages using TRIzol reagent (Invitrogen, Carlsbad, CA) as described previously (Li et al. 2011). The titer of DWV and expression of Vg were quantified by one-step SYBR Green real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The expression of a housekeeping gene, β-actin, in each sample was also measured for normalization of real time qRT-PCR results. The primer pairs for DWV, Vg, and β-actin were previously reported (Prisco et al. 2011). RT-PCR reactions were carried out in a 50-μl reaction volume, containing 25 μl of 2× Brilliant SYBR Green QRT-PCR Master Mix (Stratagene, LA Jolla, CA), 0.4 μM each of forward and reverse primers, and 1 μg of template RNA. The thermal profile for the one-step RT-PCR was as follows: one cycle at 50°C for 30 min, one cycle at 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s. After amplification, a dissociation curve was constructed using 81 complete cycles of incubation where the temperature was increased by 0.5°C per cycle, beginning at 55°C and ending at 95°C to verify presence of a single product. Negative controls (no reverse transcriptase and no template) were included in each run of the reaction and yielded no products.

qRT-PCR was replicated three times for each sample to address the variability of the analysis process. For both DWV and Vg, the values of the cycle threshold (Ct) obtained from individual bees from each cage design after β-actin normalization were averaged, and the data are represented as mean ± SE. The comparison of the relative amount of DWV and Vg in different cages was conducted by using the comparative Ct method ($2^{-\Delta\Delta Ct}$ Method), as described previously (Li et al. 2011), after confirmation of approximately equal amplifying efficiencies of DWV, Vg, and β-actin. The concentration of DWV and Vg in each cage was calculated using the formula $2^{-\Delta\Delta Ct}$ and expressed as the fold change relative to the calibrator, the cage that had the minimal level of DWV or Vg.

Diet Comparison. The combination of Cage-5 and Feeder-2, which displayed the best performance by supporting longest survival for bees, was used for the further comparison of diets for provisioning caged bees. Newly emerged bees from the same colonies were transferred into eight cages (30 bees per cage) that were divided into two groups. One group of bees was supplied only with sucrose syrup and another group was fed with both syrup and bee bread, a combination of honey and pollen. Bee bread was freshly collected from hoarding cells, and was slightly ground into powder in a mortar. To minimize the effect of any microorganisms in bee bread, ground bee bread was spread on a tray and treated for 1 h with ultraviolet light, and it was agitated evenly once after half an hour treatment. It was loaded into 1.5-ml tubes and stored at −20°C until use. When being tested, bee bread was replaced every other day to prevent drying and microbial growth. Each group was further divided into two subgroups. One subgroup was used to test effects of two different diets on bee survival by checking bee mortality every day for a 20-d observation period. Another subgroup was used to measure transcript level of Vg in bees at 5-d intervals, also for a 20-d observation period (Day-0, Day-5, Day-10, Day-15, and Day-20) after receiving different diets. The real time qRT-PCR method for quantifying Vg expression level was the same for DWV quantification as described above.

Statistical Analysis. Cumulative mortality rate was calculated by summing dead bees at point-in-time mortality assessment and then dividing this number by the total number of bees introduced into each cage ($N = 30$). Data are expressed as mean ± SD from three replications for each cage or feeder and then normalized by arcsine-square-root transformation of percentages. The transformed arcsine square root percentages were subjected to the Tukey–Kramer honestly significant difference (HSD) test for effects of different cages, feeders, and diets. A P value of <0.05 was considered statistically significant.

Results

Survival of Worker Bees in Relation to Cages. The bees kept in each of the different cages exhibited significantly different mortality rates (Fig. 3). The bees in Cage-5 displayed the longest survival curve among all examined cages. Adult honey bees in Cage-5 had an average survival of 37 d, followed by 33 d in Cage-8 and Cage-7, and 31 d in Cage-1 and Cage-2. By day 22, when at least one group of bees had reached a peak mortality of 100%, the cumulative mortality of bees in Cage-5 was only 50 ± 19%, followed by 68 ± 28% in Cage-8, and 72 ± 18% in Cage-1. The cumulative mortality of bees at day 22 in Cage-5 was significantly lower than bees in other cage groups ($F = 2.226$; df = 10, 20; $P = 0.016$).

Survival of Bees in Relation to Feeders. The bee mortality curves for different feeders (all used with Cage-5) showed that Feeder-2 supported the best survival of caged bees (Fig. 4). When bee mortality reached 100% at day 15 in Feeder-6, the mortalities were 13 ± 3, 14 ± 5, 17 ± 7, 18 ± 3, and 85 ± 4% using Feeder-2, Feeder-4, Feeder-1, Feeder-3, and Feeder-5, respectively. The mortalities in cages having Feeder-5 and Feeder-6 were significantly greater than those in cages having the other four feeders ($F =$

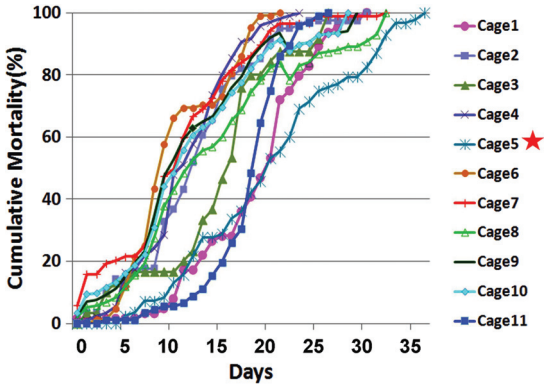

Fig. 3. Average cumulative mortality of bees kept in different cages. The bees in Cage-5 (highlighted by a star) displayed the longest survival curve among all examined cages. To improve visualization, the error bars at each time point are not shown in the figure.

29.035; df = 5,12; $P = 0.001$). However, the cumulative mortalities for bees given Feeder-1, Feeder-2, Feeder-3, and Feeder-4 were not different at day 15 ($F = 0.104$; df = 3, 8; $P = 0.956$). At day 19, when 100% bee mortality was attained using Feeder-5, bee mortalities were 58, 66, 75, and 80% using Feeder-2, Feeder-1, Feeder-4, and Feeder-3, respectively; There were no significant differences in bee mortality among these four feeders ($F = 0.562$; df = 3, 8; $P = 0.566$).

The high mortality rate of bees observed in the cages using Feeder-5 and Feeder-6 was likely because of leakage of the sugar solution from the feeders. Feeder-6 had a significantly higher quantity of leaked sugar syrup (Fig. 5). Feeder-5 also had a significantly larger quantity of leaked sugar syrup on day 5 of observation. The leaking could cause direct food loss and mold growth inside of the cage. If leaking syrup gets on a bee, this may lead to abnormal death.

DWV Titer and Vg Transcript Level in Bees in Relation to Cages. After confirmation of the same amplification efficiencies for DWV and β-actin, the average concentrations of DWV titer in bees from different cages were analyzed. Bees kept in Cage-3

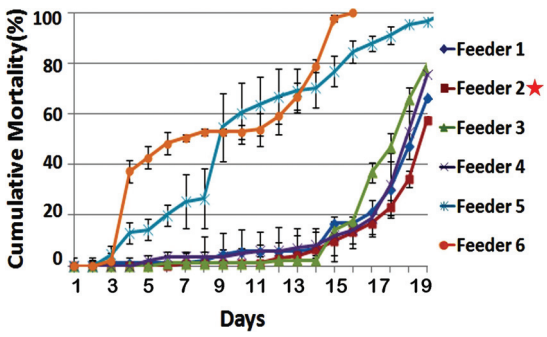

Fig. 4. Average cumulative mortality of worker bees using the same cage design (Cage-5) but different feeders. Feeder-2 displayed the best result in supporting the survival of caged bees.

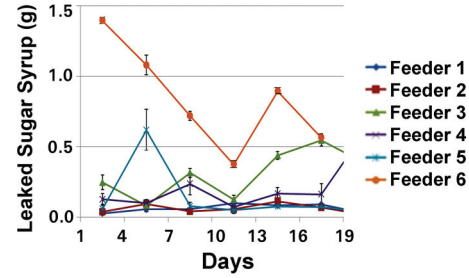

Fig. 5. Weight of sugar syrup leaked from different feeders onto the paper towel placed under the bottom of the cages.

had the lowest average DWV titers, and was therefore chosen to be the calibrator. The concentration of DWV RNA in bees from other cages was compared with that of Cage-3 and expressed as n-fold change. Compared with bees in Cage-3, the concentration of DWV in bees from different cages varied significantly and the difference in DWV concentration ranged from 2.2-fold to 2977-fold (Fig. 6). The concentrations of DWV in bees from Cage-5, Cage-8, Cage-10, and Cage-11 were significantly lower than that of bees from other cages ($F = 5.656$; df = 10, 59; $P = 0.001$), reflecting different levels of virus replication under different cage conditions.

Quantification of Vg transcript of bees from different cages yielded completely different results in different trials (data not shown). The inconsistent results reflect the inherent variability of individual bees and suggest that there is no correlation between the level of Vg expression and containment conditions for bees.

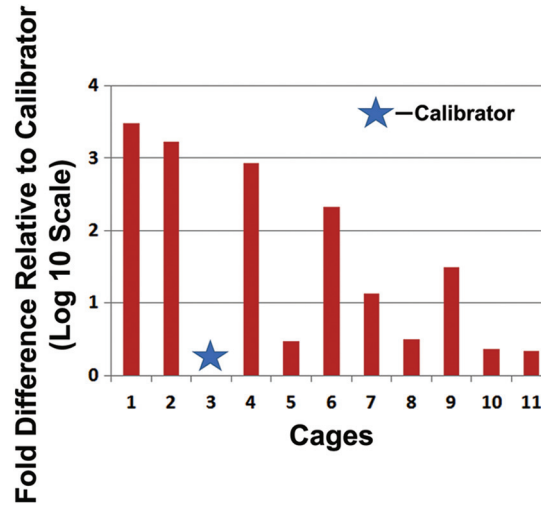

Fig. 6. Relative abundance of DWV RNA genome copies in bees from different cages. Bees kept in Cage-3 had the lowest level of DWV titer and therefore Cage-3 was chosen as a calibrator. The concentration of DWV RNA in bees from other cages was compared with the calibrator and expressed as n-fold change. The y-axis depicts Log 10 fold change relative to the calibrator.

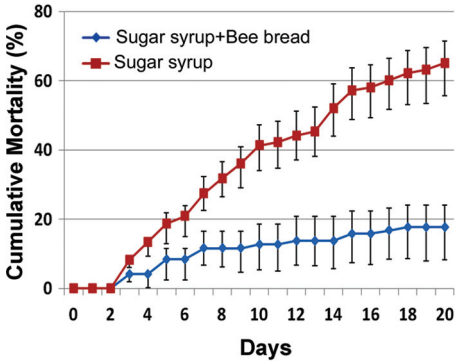

Fig. 7. The cumulative mortality of worker bees in the group fed with sugar syrup and the group fed with a combination of sugar syrup and bee bread.

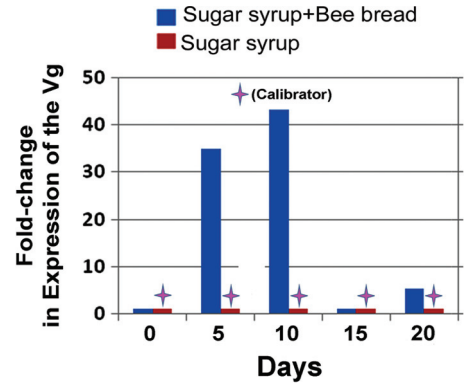

Fig. 8. Fold changes in expression of the Vg relative to the calibrator. The bees fed with sugar syrup had relatively lower level of Vg expression compared with bees fed with sugar syrup and bee bread and therefore was chosen as a calibrator. The level of Vg expression in bees fed with sugar syrup and bee bread was expressed as the fold change relative to the calibrator at each time point of observation.

Survival of Bees and Level of Vg Expression in Relation to Diet. Analysis of the effects of diet on the survival of caged bees indicated that addition of bee bread, a protein source, could significantly increase the survival of caged bees. The difference in Vg expression levels between two groups was significant at 5 d (e.g., Student *t*-test, df = 17; $t = 4.25$; $P = 0.001$); the survival of bees fed with additional bee bread was significantly higher than that of bees only with sucrose syrup after day 8 (e.g., Student *t*-test, df = 6; $t = 2.808$; $P = 0.031$). When bees fed with only syrup reached almost 60% cumulative mortality at day 20, the cumulative mortality in the group of bees fed with a combination of syrup and bee bread was 20% less on the same date (e.g., Student *t*-test, df = 6; $t = 3.976$; $P = 0.007$; Fig. 7).

The addition of bee bread increased Vg expression in caged bees. Five days after caged bees were supplied with bee bread, their Vg expression was 34.96-fold higher than that of bees fed only with sucrose syrup. The level of Vg expression increased to 43.3-fold higher at day 10 in bees fed with bee bread than in bees fed only with syrup. However, around experimental day 12–13, caged bees were found to have stopped consuming the bee bread. The consequence was a steep decline in the level of Vg expression observed at day 15 and 20 (Fig. 8).

Discussion

Cages for maintaining adult worker bees under in vitro conditions have been widely used over the years for laboratory experiments to assess the impacts of various biotic and abiotic factors on the survival and development of honey bees. However, several laboratories have developed and applied different cages, making research findings difficult to compare among different groups. This international collaborative study of evaluating the performance of different cage designs represents a critical step toward improving and developing standard methods in *A. mellifera* research.

Although cages used in this study differed in shape, size, and building materials, they all possessed the following common features: 1) an entry way for introduction or removal of materials such as bees, food, water, or treatments, 2) ventilation opening(s) for free circulation of air, 3) at least one side of sliding glass or transparent mesh fiberglass that allows for a clear view into the cage to easily count and observe bees at all times, and 4) disposable or washable so as to avoid contamination across trials. The evaluation showed that there was variation in survival and physiological conditions of bees among cage types. Bees kept in Cage-5, Cage-8, and Cage-7 had significantly higher survival, higher levels of Vg expression, and lower DWV titer than bees kept in other cages. The evaluation of different feeders individually inserted into Cage-5 indicated that a well-performing feeder was an integral part of an effective cage system, and that the bees could benefit significantly from having a steady food supply from their feeders. A good feeder was found to be characterized by the ability to form sufficient surface tension to prevent sucrose syrup from leaking or dripping out, but allows bees to drink the liquid. Feeder-2, modified from a 20-ml plastic syringe, most strongly possessed this feature, and did not leak like some of the bottle or tube feeders (Li et al. 2011).

The bees from cages that exhibited high levels of survival had relatively lower titers of DWV, suggesting that DWV may have been killing bees, or at least is a significant marker reflecting stress level and health status of the host. Bees kept in Cage- 3, resembling traditional queen cages, had a moderate level of survival but the lowest DWV titer. The small size of this cage, however, limits its application when tests require more bees. Queen cages are advantageous for postal transmission of queens and attendants (Bigio et al. 2012).

The addition of bee bread, a combination of pollen and honey that is the natural source of proteins to honey bees, was shown to significantly improve

bees' longevity and enhance the level of Vg expression of caged bees. This result is consistent with previous findings that pollen nutrition is a key factor influencing honey bees immune function, development, and longevity (Rinderer et al. 1974, Alaux et al. 2010, Brodschneider and Crailsheim 2010, DeGrandi-Hoffman et al. 2010, Alaux et al. 2011). Vg is a yolk precursor protein and synthesized depending on pollen consumption in honey bees (Raikhel and Dhadialla 1992). Aside from having a reproductive function, Vg plays important role in promoting bee immunity and longevity (Amdam et al. 2004, 2005, 2006; Seehuus et al. 2006). The enhanced level of Vg reflects the physiological robustness of individual bees feeding on bee bread, which in turn could lead to increased immune responses and improved longevity in bees.

There was noticeable interaction between the impacts of the cage, feeder, and diet on bee health. A combination of Cage-5, Feeder-2, and a diet consisting of sucrose syrup and bee bread was proven to be the most satisfactory for improving survival of caged bees. When 100% mortality was achieved in a few other cages at day 19–20, the mortality was only 10% for bees kept in the combination of Cage-5, Feeder-2, and bee bread diet. As a result, we would conclude that this combination of Cage-5 or Cage-8 or Cage-7, Feeder-2, and diet consisting of sugar syrup and bee bread could have important implications for improving care of adult honey bees for future laboratory experiments.

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